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Localization of a Novel Adhesion-Promoting Site on Acetylcholinesterase Using Catalytic Antiacetylcholinesterase Antibodies Displaying Cholinesterase-like Activity

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Abstract

A monoclonal antibody (MAb) raised against human acetylcholinesterase was found to have catalytic activity. A similar phenomenon was observed in a polyclonal antibody raised against the same antigen. The antibodies were demonstrated to be pure, and no contamination with either acetylcholinesterase or butyrylcholinesterase was found. Both antibodies hydrolyzed acetylthiocholine, an acetylcholinesterase substrate, and the MAb followed Michaelis–Menten kinetics. Six other MAbs and one other polyclonal antibody showed no evidence of catalytic activity.

Acetylcholinesterase is a key component in the transmission of the nerve impulse, and is also expressed nonsynaptically during embryonic development, and abnormalities in expression are seen in neural tumors and degenerative disorders. This unusual expression is believed to be associated with a novel function of the enzyme related to differentiation and cell adhesion. Autoantibodies to acetylcholinesterase have been observed in a variety of neurologic, muscular, and autoimmune disorders.

In an investigation of the possible role of acetylcholinesterase in cell adhesion, we showed that the enzyme promoted neurite outgrowth in neuroblastoma cell lines, and conversely, that certain antiacetylcolinesterase antibodies abrogated cell–substrate adhesion. Interestingly, the antibodies most effective in this regard were catalytic. Preliminary epitope analysis indicated a conformational epitope in the N-terminal domain. This domain contains the active site, within a deep gorge, and the peripheral anionic site at the rim of the gorge. Peripheral-site inhibitors, but not active-site inhibitors, also interfered with adhesion, and competed with the catalytic monoclonal

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binding to acetylcholinesterase, indicating that the epitope recognized is associated with the peripheral anionic site. The inhibitor data also support the supposition that catalysis in these antibodies may have arisen from stable complexation of acetylcholinesterase with an inhibitor.

We conclude that the catalytic antiacetylcholinesterase antibody interacts with structures associated with the peripheral anionic site, thus defining a novel site on the molecule involved in cell adhesion. This finding has implications for our understanding of the potential importance of this peripheral site in a variety of congenital, neoplastic, and degenerative conditions.

Index Entries: Catalytic antibody; acetylcholinesterase; cell adhesion; peripheral anionic site.

Introduction

The principal biological role of acetylcholinesterase (EC 3.1.1.7; AChE) is the hydrolysis of the neurotransmitter acetylcholine in the synapse, resulting in termination of the nerve impulse. It has been described as an evolutionarily perfect enzyme *(1)*; it has a turnover number of 1.6×10^4 s^{-1} for the reaction with acetylcholine *(2)*; and it operates at close to the rate of substrate diffusion. There is also evidence of novel noncatalytic functions in neurogenesis and differentiation *(3–7)* and neurodegeneration *(8)*, as well as in haematopoietic cell development *(9)* and the etiology of leukemias *(10)*.

The crystal structure of AChE has been determined *(11)*, providing explanations for the high catalytic efficiency and the interactions of AChE with its numerous inhibitors. The active site lies within a deep gorge—20 Å in depth—lined with hydrophobic residues. The site itself comprises the esteratic subsite—a Ser, His, Glu catalytic triad typical of the serine proteases—and an "anionic" subsite that binds the quaternary ammonium group of acetylcholine. At the rim of the gorge, about 14 Å away from the active site, lies a second ammonium-binding site—the peripheral anionic site—that appears to be responsible for substrate inhibition *(12)*.

AChE catalysis occurs through an acylenzyme mechanism involving nucleophilic (serine) and general acid–base (histidine) functions. The acyltransfer reaction is not a particularly difficult one to mimic, as shown by the fact that the first catalytic antibodies demonstrated were those with esterase-like activity *(13,14)*. Previously, Kohen et al. *(15,16)* had observed naturally occurring autoantibodies with esterase activity. Joron et al. *(17)* and Izadyer et al. *(18)* have demonstrated AChE-like activity in antiidiotypic antibodies raised against the anti-AChE monoclonal AE-2, which recognizes active-site structures.

In this article, we describe a catalytic MAb raised against AChE that showed cholinesterase-like activity. We also show how this antibody has proven to be instrumental in identifying the noncholinergic site on AChE involved in neurogenesis.

Methods

Production and Purification of Antibodies

MAbs were raised according to standard protocols *(19)*. Balb/C mice were inoculated subcutaneously and then intraperitoneally with 90 μg human erythrocyte AChE (Sigma) in Freund's complete adjuvant. Care and handling of animals was managed in accordance with the guidelines set forth by the Animal Research Review Committee of the University. Fusion with the SP2/Ag0 nonsecreting mouse-myeloma cell line was performed. The resulting anti-AChE-producing hybridomas were identified by ELISA using human erythrocyte AChE as antigen and biotinylated antimouse IgG and streptavidin-peroxidase (Kirkegaardt Perry Laboratories, Gaithersburg, MD). Positive hybridomas were cloned twice by limiting dilution onto mouse peritoneal macrophage-feeder layers and expanded, and the antibody was harvested from tissue-culture medium or ascites fluid from mice primed with 0.5 mL Pristane (2,6,10,14-tetramethyl pentadecane; Sigma) 1 wk before intraperitoneal injection of 10^6 hybridoma cells.

Polyclonal antibodies against AChE were raised in two rabbits by injection of 180 μg human erythrocyte AChE in adjuvant, in the subscapular region. Rabbits were subsequently venesected and the serum was collected, aliquotted, and stored in the presence of 0.1% NaN_3. Four additional MAbs were donated from Dr. Mary K. Gentry of the Walter Reed Army Institute, Washington, DC.

Antibodies from ascites fluid or tissue-culture medium were purified on Protein A-Sepharose using low-salt buffers (*19*). IgG-containing fractions were identified and quantitated by absorbance at 280 nm and ELISA using biotinylated antimouse IgG (Kirkegaardt Perry Laboratories) as probe.

Measurement of AChE Enzyme Activity

AChE enzyme activity was measured by the Ellman assay *(20)* using 0.5 m*M* acetylthiocholine as substrate and the production of the yellow nitrobenzoate anion from 1 m*M* dithiobisbenzioc acid as detection. Ethopropazine (1.5 μ*M*; Sigma) was used as a specific butyrylcholinesterase inhibitor.

Sucrose Density-Gradient Sedimentation

Samples of affinity-purified IgG, AChE, and mixtures of the two were applied to 5–20% (w/v) sucrose density gradients in 50 m*M* Tris-HCl, pH 7.4, containing 0.1% Triton X-100 and 1 *M* NaCl, and centrifuged for 20 h at 97,000*g* at 4°C (Beckman SW 41 rotor). Gradients were calibrated by inclusion of markers of known sedimentation values (bovine serum albumin, 4.65S; fibrinogen, 7.63S; and catalase, 11.2S) and were also measured by direct densitometry. The gradients were fractionated into approx

40 fractions of 0.25 mL each. Fractions were scanned at 280 nm and assayed for IgG and AChE by ELISA, as above, and for cholinesterase activity by the Ellman assay.

Kinetic Measurements

MAb or enzyme (12 μ*M*) was incubated in buffer (50 m*M* Tris-HCl, pH 7.6) containing 1 m*M* dithiobisbenzoic acid at 25°C. Temperature and pH were monitored to ensure that no variation occurred. The reactions were initiated by addition of varying amounts of the substrate (acetylthiocholine) to give 2–8000 μ*M* final concentration. Reactions were monitored spectrophotometrically by the increase in absorbance at 410 nm. The first-order rate constant (spontaneous hydrolysis), measured in the absence of antibody or enzyme, was used to correct the initial rate data.

Cell Experiments

The human neuroblastoma cell lines, N2α, GIMEN and Kelly, were cultured in Dulbecco's MEM with 10% fetal calf serum (Highveld Biological, Johannesburg) at 5% CO_2. Cells were plated at 2×10^4/mL in 96-well plates (Nunc, Denmark) and anti-AChE antibodies were added to the wells at concentrations of 32, 16, 8, and 4 μg IgG/mL. Where azide had been included in the antibody solution, an equivalent concentration of azide was added to control wells. Cell adhesion was measured as follows: unattached cells were removed from triplicate wells by pipeting, and retained. Each well was then rinsed with 0.2 mL phosphate-buffered saline, and the rinsing solution was combined with the unattached cells and counted in a hemocytometer. The remaining attached cells were removed with Versene, and counted. These two values give the total numbers of attached and unattached cells in the well, respectively. The number of attached cells was expressed as a percentage of the total (attached + unattached) and described as the "percentage adherent."

Cholinergic inhibitors were also added to the cells in certain experiments, as follows: BW284c51 (1,5 *bis*-(allyldimethyl ammonium phenyl) pentan-3-one dibromide (Sigma) at 316, 158, 79, and 40 μ*M*, eserine (physostigmine, Sigma) at 1.16, 0.58, 0.29, and 0.14 m*M*, edrophonium, and propidium iodide (both Sigma) at 450, 225, 112.5, and 56 μ*M*.

Competition ELISA

Ninety-six-well microtitre plates were coated with 143 n*M* AChE in 50 m*M* sodium carbonate, pH 9.25 (calculations based on a 70-kDa AChE monomer, containing one active site). The cholinergic inhibitors—propidium iodide, BW284c51 and edrophonium—were used in a range of concentrations between 0 and 400 μ*M*, and incubated with the AChE for 2 h at room temperature. After removal of the inhibitor solutions, MAbs E8, E413D8, and G9 were added at a concentration of 560 n*M* (four times the AChE active-site concentration) and incubated for 2.5 h, also at room tem-

perature. The amount of MAb bound was probed with biotinylated anti-mouse IgG (Sigma) and peroxidase-streptavidin (Zymed, San Francisco, CA). The blocking solution was 0.5% bovine serum albumin in phosphate-buffered saline, which was also used for dilution of reagents. The washing solution was 0.1% Triton X-100 in phosphate-buffered saline.

Fluorescence Measurements

Fluorescence was measured in a Perkin Elmer fluorometer, coupled to a computer. Measurements were carried out in 300 μL solution volume, in 1.0-mL semimicro cuvets. The buffer used was 100 m*M* Tris-HCl, pH 8.0. 250 n*M* AChE was incubated overnight at 4°C with MAb concentrations of 0–1 μ*M*; 750 n*M* propidium iodide *(21)* was added 10 min before fluorescence measurement. The excitation wavelength was 535 nm, and that of emission, 602 nm.

Statistics

Statistical significance was calculated with Student's *t*-test for two-tailed samples.

Results

Detection of Catalytic Activity in Antibodies

Catalytic behavior of the MAb E8 was first observed in an assay designed to detect catalytic activity in AChE after reaction with various MAbs. The wells containing E8 showed consistently high levels of AChE-like activity. This was far higher than could be accounted for by the amount of AChE present or by an enhancement of AChE function (*see* Fig. 1). This catalytic behavior of MAb E8 was identified in all batches of purified antibody, tissue culture medium, and ascites fluid. The cholinesterase-like activity eluted with the IgG fraction. Further ELISA measurements with three anti-AChE antibodies confirmed that the fraction was indeed IgG, and was not recognized as AChE. Inclusion of ethopropazine—a specific butyrylcholinesterase inhibitor—in the assay had no effect on activity of the IgG fraction. This indicated that the observed activity was not caused by contaminating BChE, and was not BChE-like.

Similar catalytic behavior was observed in one out of two polyclonal antibodies, raised in rabbit against human erythrocyte AChE. Because of the lack of specificity of polyclonal antibodies *(22)*, no further purifications or measurements were done.

Demonstration of Antibody Purity

The major concern was that MAb E8 and the polyclonal antibody had become contaminated with AChE itself. Demonstration of IgG purity on polyacrylamide gels is of little value in this case, as the denaturing conditions would probably result in the breaking of any antibody–AChE com-

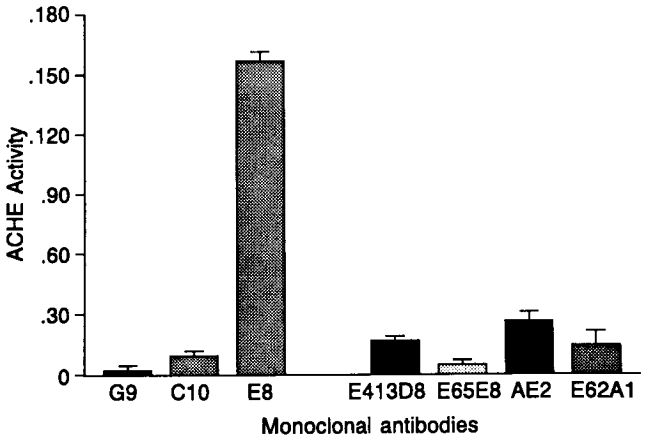

Fig. 1. Cholinesterase-like activity of MAb. The three MAbs on the left-hand side are those raised in our laboratory (*see* Methods). Cholinesterase activity was measured by the Ellman assay *(21)* and is expressed as the OD at 405 nm.

plex. In addition, AChE is located in a very similar position to IgG on SDS-PAGE. It was therefore decided to use sucrose-density gradients (5–20%), which would be capable of separating IgG itself from any IgG complexed to AChE.

Separate gradients containing E8-IgG—with and without AChE—irrelevant IgG—with and without AChE—and AChE itself were run. It was observed that the IgG fractions produced a single peak, identified by A280 and by the anti-IgG ELISA. This peak was not recognized as AChE by three different anti-AChE antibodies (*see* Fig. 2a). In the case of MAb E8, it had cholinesterase-like activity. In contrast, when AChE was added to the IgG sample on the second gradient, the IgG peak shifted to higher density (*see* Fig. 2c). Four peaks were noted: the first, at fractions 7-8, corresponds to the IgG position, and was recognized by anti-IgG but not by anti-AChE antibodies. The second peak, at fractions 15-16, was identified by anti-AChE antibodies and also the enzyme assay, and corresponds to uncomplexed AChE. The third peak, at fraction 24, was identified by all three assays, and probably represents AChE complexed with a single IgG molecule. The last peak, at fraction 32, was likewise recognized by all three assays, and may represent a complex of two AChE with one IgG molecule. Alternatively—because of the similarity in molecular weight between AChE and IgG, and the fact that the catalytic antibody recognizes the anionic sites of AChE, which are separated by 14 Å—the peak may comprise one AChE and two IgG molecules. A similar sedimentation profile was observed when the polyclonal antibody was run.

Kinetic Behavior of MAb E8

Kinetic measurements on MAb E8 were carried out, using acetylthiocholine as substrate and *Torpedo californica* AChE as comparison. The

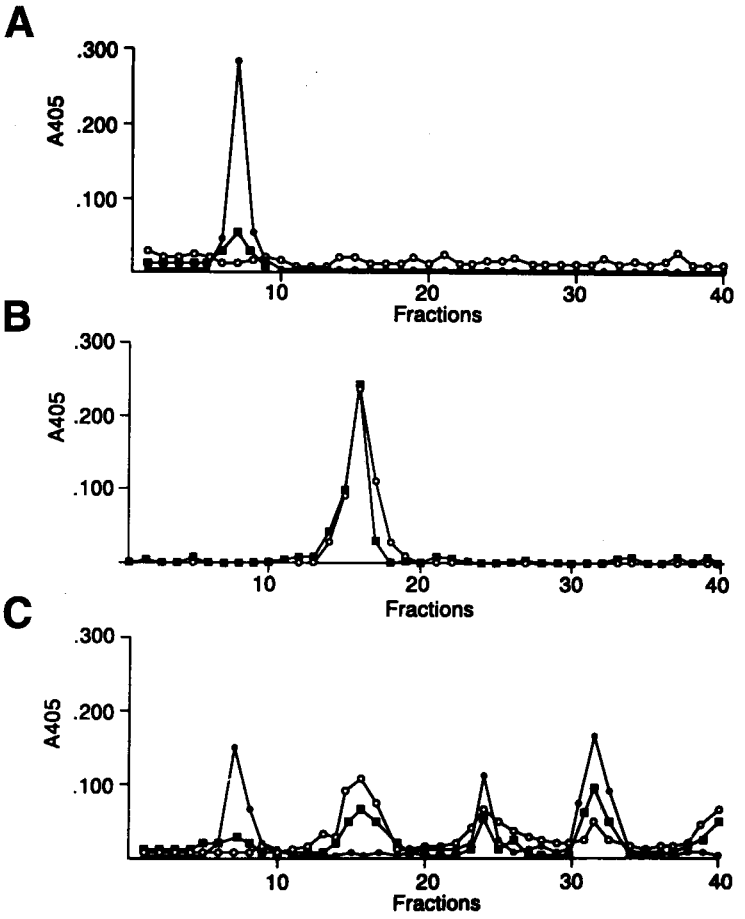

Fig. 2. Density-gradient sedimentation of MAb E8. **(A)** MAb E8 alone; **(B)** AChE alone; **(C)** MAb E8 with the addition of 650 μg human erythrocyte AChE. IgG: (●--●); AChE immunoassay: (○--○) AChE enzyme assay (□--□).

Michaelis–Menten constant, K_m, was determined from the *x* intercept on the double reciprocal Lineweaver–Burk plot (*see* Fig. 3). MAb E8 showed characteristic enzyme behavior, and yielded a K_m of 29 ± 6 μ*M* compared to a K_m of 0.65 ± 0.11 μ*M* for *Torpedo* AChE.

The Effects of Antibodies and Inhibitors on Neuroblastoma Cells In Vitro

Some, but not all, of the panel of anti-AChE antibodies—when added directly to the culture medium of the cells—inhibited cell–substrate adhesion (*see* Table 1). The results are expressed for the highest concentration of antibody (40 μg/mL); a dose-dependent effect was noted at the lower concentrations. The cells remained rounded and in suspension; if the antibodies were added after the cells had been plated, the latter rounded up and detached from the flask. Addition of antibodies to cells that were

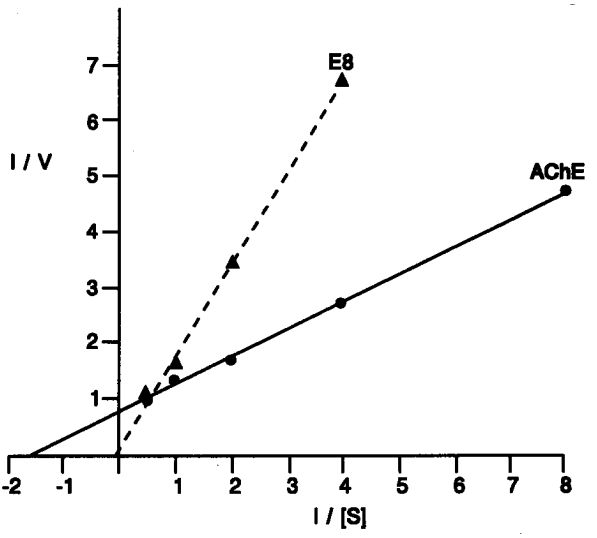

Fig. 3. Lineweaver–Burk plot for MAb E8 and *Torpedo* AChE. MAb or enzyme (12 μ*M*) was incubated in buffer (50 m*M* Tris/HCl, pH 7.6) containing 1 m*M* dithiobisbenzoic acid, at 25°C. The reactions were initiated by addition of various amounts of the substrate (acetylthiocholine) to give 2–8000 μ*M* final concentration.

already differentiating resulted in a retraction of neurites and a reversal of the differentiated morphology. These effects were visible after 24 h incubation.

Interestingly, the antibodies most effective in inhibiting adhesion were the catalytic ones: only 8% of cells incubated with MAb E8 were adherent, and in the case of the polyclonal, PCW, this figure was reduced to zero (control = 96.5%). Both these results are highly significant ($p < 0.001$). Partial inhibition of adhesion was seen with the MAbs E62A1 and E65E8, and with the other polyclonal antibody (*see* Table 1).

A similar effect on cell adhesion was observed after incubation with certain anti-AChE inhibitors (*see* Table 2). The inhibitors most effective in this regard were BW284c51 (20.2% cells adherent) and propidium (29.6% cells adherent). These results are markedly different from the control ($p < 0.001$). Eserine and edrophonium did not, however, affect adhesion.

Competition Between Antibodies and Inhibitors

It appears (*see* Fig. 4) that MAb E8 competes with the peripheral-site inhibitors BW284c51 and propidium—but not with active-site inhibitors edrophonium and eserine—for a site on the AChE molecule. Setting the amount of antibody bound in the absence of inhibitor as 100%, only 36.87 ± 7.91% bound in the presence of 390 μ*M* BW284c51, and 26.15 (6.99% in the presence of the same concentration of propidium. The differences between these values and the controls are significant ($p < 0.01$). In contrast, in the presence of the anionic-site inhibitor edrophonium, 87.69 ± 4.76%

Table 1
Effects of Anti-AChE Antibodies on Neuroblastoma Cell Adhesion[a]

Antibody	Percentage Adherent Cells
E413D8	93.4 ± 2.1
AE-2	95.6 ± 2.7
E62A1	41.2 ± 8.5
E8	8.0 ± 3.6
G9	94.1 ± 2.7
C10	94.7 ± 2.5
E65E8	54.3 ± 7.9
PCW	0.0 ± 0.0
PCB	71.8 ± 4.9
Control	96.5 ± 2.3

[a]The antibodies PCW and PCB are polyclonals. Results are shown as means and standard deviations, after 4 d incubation.

Table 2
Effects of Cholinergic Inhibitors on Neuroblastoma Cell Adhesion[a]

Inhibitor	Percentage adherent cells
BW284c51	20.2 ± 4.3
Edrophonium	87.2 ± 5.9
Eserine	95.5 ± 3.2
Propidium	29.6 ± 7.1
Control	96.1 ± 3.7

[a]Results are given as means and standard deviations, after 5 d incubation.

of antibody bound to AChE. A comparison with the noncatalytic, nonadhesion-inhibiting MAb E413D8 yielded 96.17 ± 2.79%, 97.59 ± 1.25%, and 94.80 ± 2.78% for BW284c51, propidium, and edrophonium, respectively.

Propidium exhibits a 10-fold increase in fluorescence on binding to AChE (*21*), making it a useful probe for competitive ligand binding to the enzyme. In the presence of 1 μ*M* E8, only 26.38 ± 5.11% of propidium bound, as opposed to 98.43 ± 1.22% in the case of the noncatalytic antibody (*see* Fig. 5). Again, these results are significant ($p < 0.001$).

Discussion

It appears from our studies that two anti-AChE antibodies, the MAb E8, and one polyclonal antibody all have cholinesterase-like activity. Other anti-AChE antibodies tested did not demonstrate this phenomenon. It is of vital importance to demonstrate purity when dealing with an antibody

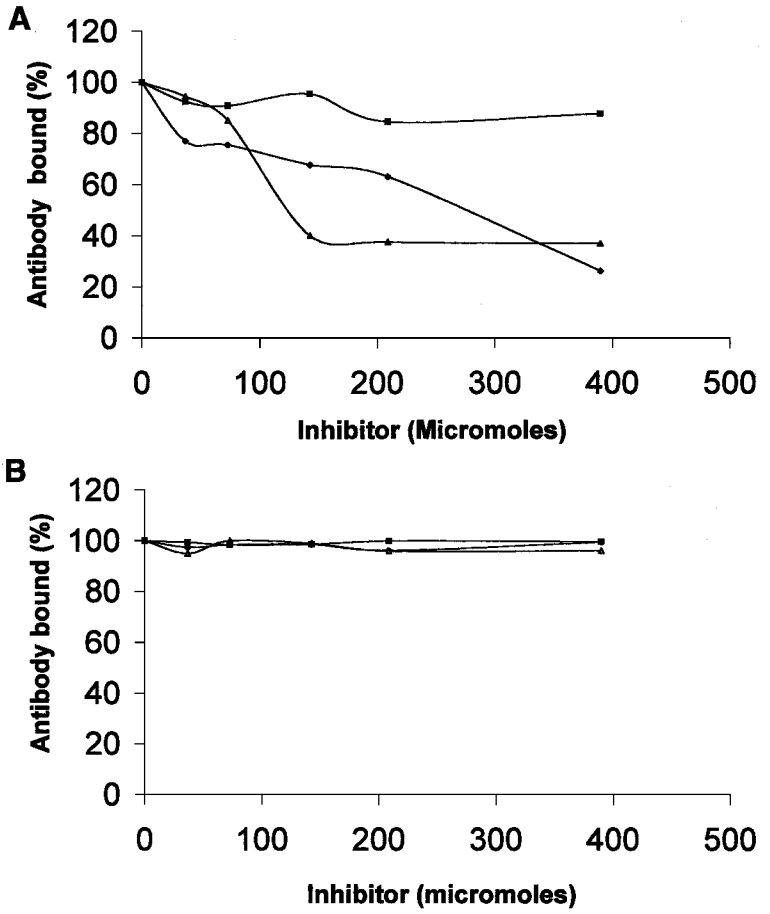

Fig. 4. Competition ELISA between MAbs and cholinergic inhibitors. **(A)** MAb E8 and edrophonium (□--□), propidium (△--△) and BW284c51 (◆--◆) **(B)** MAb E413D8 and edrophonium (□--□), propidium (△--△) and BW284c51 (◆--◆).

that catalyzes a known enzymic reaction, particularly where the antibody behaves like the antigen to which it was raised. The evidence for purity is as follows: the catalytic activity was present in all batches of tissue, culture medium, and ascites fluid, in the case of E8; and of serum, in the case of the polyclonal. The various batches had also been harvested and purified at different times. Other anti-AChE antibodies that were identically prepared and purified showed no evidence of significant catalytic activity. The activity was retained after Protein-A immunoadsorption and elution with low-pH buffers, and it coeluted with the IgG fraction.

Sucrose density-gradient sedimentation conclusively demonstrated that the IgG fraction was pure: the fraction was identified by anti-IgG antibodies, but not recognized by anti-AChE antibodies. Addition of AChE to the fractions clearly showed a significant shift in the position of IgG to approx 11 and 16S. Inclusion of the specific butyrylcholinesterase inhibitor—

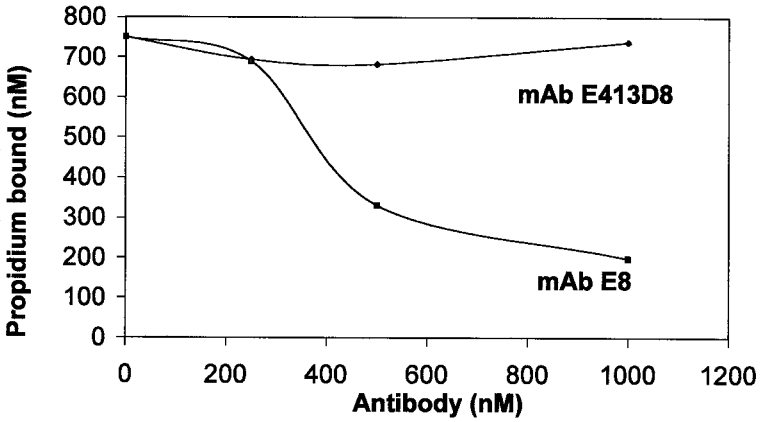

Fig. 5. Fluorescence measurement of competition between MAbs E8 and E413D8 and propidium.

ethopropazine—in the assay indicated that the catalytic activity was not caused by contamination with this cholinesterase. Furthermore, butyrylcholinesterase is very similar in both primary and oligomeric structure to AChE, and sediments in a similar manner on sucrose. A variety of anti-AChE antibodies, both monoclonal and polyclonal, failed to identify AChE in the antibody fractions. Lastly, the K_m values of E8 and AChE are different—that of E8 being 45 times greater than that of AChE.

The obvious question is how antibodies raised against AChE can show similar—yet considerably weaker—properties of the immunogen itself. We were puzzled by the very low specific activity of the human erythrocyte AChE used as immunogen (0.25–1.00 U/mg) in comparison with both the almost structurally identical *T. californica* enzyme (1000–2000 U/mg) and with published activities of human erythrocyte AChE purified in a variety of laboratories (3800–5000 U/mg *[23–25]*). Information obtained from the supplier indicated that their erythrocyte AChE was purified by the method of Niday et al. *(26)*. This method involves the use of tetramethyl ammonium chloride—a cholinomimetic compound that binds to the anionic subsite and inactivates AChE—in the purification process.

Although AChE is an enzyme of very great catalytic power, it is relatively nonspecific and can hydrolyze a variety of substrates. Its promiscuity also accounts for the wide variety of inhibitors—quasisubstrates, such as organophosphate pesticides and nerve gases, that bind to the active site without subsequent hydrolysis; and cholinomimetic compounds, with similar quaternary ammonium groups to the choline moiety of acetylcholine, that are capable of binding to both anionic sites. Tetramethyl ammonium chloride falls into the latter group. It is possible that complexation with tetramethyl ammonium chloride may create a new site that resembles an immunogenic ground state or transition state of acetylcholine by altering the steric conformation of the active site of AChE. It should also

be kept in mind that organophosphate inhibitors, which bind covalently and irreversibly to the active site, are—in this bound state—transition-state analogs of acetylcholine *(27)*. Such complexation may occur either in vitro during mixing with the adjuvant or in vivo during uptake by the macrophage and antigen presentation. Such an antibody–inhibitor complex would be stable over sufficient time to allow for the generation of an antibody. These hypotheses must still be tested.

Although the synaptic role of AChE is well-known and well-characterized, it has become apparent that the molecule has a secondary function as well. This is indicated by its nonsynaptic expression *(28)*, its homology to cell adhesion and neurotrophic molecules *(29–31)*, and by direct evidence linking it to cell adhesion and differentiation *(3–7)*. We have also demonstrated the stimulation of neurite outgrowth by AChE in human neuroblastoma cells (Johnson and Moore, submitted for publication). This secondary function is noncholinergic, and therefore associated with a structural site on the molecule.

We observed that the peripheral-site inhibitors (BW284c51 and propidium) and not the active (eserine) or anionic site (edrophonium) inhibitors abrogated adhesion. All these inhibitors, with the exception of eserine—which also interacts with butyrylcholinesterase—are specific for AChE, and are expected to act on the AChE expressed on the cell surface. This suggests the involvement of the peripheral site in AChE-mediated adhesion. This adhesion-inhibiting effect of BW284c51 has been noted in other cells *(4,5)*, and there have been suggestions, based on its observed interference with both neural regeneration in *Aplysia (32)* and with the deposition of β-amyloid fibrils in Alzheimer's disease *(33)* that the AChE secondary function may be linked to the peripheral anionic site. The observation that the catalytic antibodies were most effective at inhibiting adhesion, our supposition that these antibodies are recognizing epitopes associated with active-site structures, led us to investigate direct competition between the antibodies and the inhibitors. Results from both the competition ELISA and fluorescence measurements indicated that such competition does indeed exist. We therefore conclude that the epitope recognized by E8—which appears to be necessary for neuroblastoma cell adhesion—is located at the peripheral anionic site.

In conclusion, we have described two antibodies—a monoclonal and a polyclonal—raised against human erythrocyte AChE, that show cholinesterase activity and function as weak versions of AChE itself. It is hypothesized that this occurred by complexation of AChE with an inhibitor, acting either as a substrate or transition-state analog, during antigen presentation. The antibody idiotope therefore would have characteristics of an AChE-like active site, and the epitope recognized would incorporate active-site structures. If the inhibitor were cholinomimetic, capable of binding to both anionic and peripheral sites, the subsequent epitope is likely to be associated with these structures. The observation that antibody E8 binds to peripheral-site components bears this out. Fortuitously, anti-

body E8 has also proven to be instrumental in identifying the secondary, adhesion-promoting site on the AChE molecule.

Acknowledgments

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Discussion

Paul: Tetramethylamonium chloride: is it a transition-state analog?

Johnson: It is not.

Schowen: It is a substrate analog.

Paul: So, if your antibody is directed at this analog present as a contaminant in the enzyme, the antibody is an example of an antisubstrate antibody.

Gabibov: Did you try to inhibit your activity with an antibody to the active site of acetylcholinesterase or with known enzyme inhibitors?

Johnson: No. That is something I want to do.

Green: I think that when you attempt to publish such work there will be at least some reviewers who will want proof that there is no contaminant, and some defined pattern of inhibition of the enzyme inhibitors with the catalytic antibody. Furthermore, you could try different substrates, as you showed that there may be a different reactivity with the antibody compared to the enzyme.

Johnson: The issue of contaminants was discussed in our *European Journal of Immunology* paper in 1995. Thank you for your suggestions.